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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6: C12Q 1/68 — A1

(11) International Publication Number: WO 95/07362

(43) International Publication Date: 16 March 1995 (16.03.95)

(21) International Application Number: PCT/GB94/01967

(22) International Filing Date: 9 September 1994 (09.09.94)

(30) Priority Data:
9318751.6 9 September 1993 (09.09.93) GB

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(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: DETECTION AND SPECIATION OF *CAMPYLOBACTER*

(57) Abstract

Method for detecting *Campylobacter* by PCR detection of DNA sequence, highly conserved between species *lari, coli, jejuni* and *upsaliensis*. Speciation between these four is possible as the PCR product is differentially cleaved by restriction endonucleases.

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DETECTION AND SPECIATION OF *CAMPYLOBACTER*

This invention relates to the detection and speciation of *campylobacter* bacteria, for example in clinical, environmental and food samples. In particular, this invention relates to a method of detecting whether a sample contains *campylobacter* and to a method of differentiating between the main *campylobacter* species *jejuni, coli, upsaliensis* and *lari*.

campylobacter species are recognised as the most frequent cause of bacterial gastroenteritis in the United Kingdom and many other countries throughout the world. In the U.K. approximately 90% and 10% of case isolates are identified as *campylobacter jejuni* and *campylobacter coli* respectively, plus a small number of other species such as *campylobacter upsaliensis* and lari. The majority of the infections are sporadic the source of which remains largely unknown although the importance of several vehicles is now recognised.

There is a known desire to be able to detect and differentiate species of *campylobacter*. However, it is also known that present *campylobacter* enrichment culture techniques lack sensitivity, making detection difficult. *campylobacter jejuni* does not multiply in foodstuffs and low numbers may be present together with a high background of indigenous microflora. Also, surface viable counts of *campylobacter* can decrease rapidly and cells that are potentially culturable are often lost before samples reach a laboratory for analysis. Another factor making detection problematic is that antibiotics used in culture enrichment media may damage already weakened *campylobacter*.

There are currently available assays for detection of a variety of food and water-borne pathogens; L.pneumophila,

V.vulnificus, enteroinvasive E. *coli*, Shigella; but no satisfactory method of detecting *campylobacter* or distinguishing between the four main *campylobacter* species is known.

A method of detecting *campylobacter* has been published by Giesendorf, B A J, et al in Applied and Environmental Microbiology, December 1992, pages 3804-3808. The method detects the species *jejuni*, Coli and *lari*, and produces similar results to conventional methods but in a reduced time. The method suffers from a number of drawbacks. It does not enable detection of the species *upsaliensis*. Further, the method employs polymerise chain reaction (PCR) techniques but nevertheless requires a short enrichment culture before the PCR can be employed. Further still, the primer used for the PCR does not have the precise homology with DNA sequences in the three *campylobacter* species that can be detected using the method.

Another method for detecting *campylobacter jejuni* and *campylobacter* coli is known from Wegmuller, B E et al, Applied Environmental Microbiology, vol. 59, part 7, 1993 pages 2161-2165. The described method detects only the species *jejuni* and *coli*.

In addition to the above-identified problems with detection and speciation of *campylobacter*, recent work on *campylobacter jejuni* suggests that in certain circumstances it enters a "non-culturable, viable form" when subjected to environmental stresses, such as pH or temperature extremes, increased oxygen tension or nutrient depletion. In this form, *campylobacter* infectivity is maintained but the organisms cannot be cultured. Thus there exists a need for the improvement of methods of detection of non-culturable forms of *campylobacter*.

It is an object of this invention to provide a method of testing for the presence of *campylobacter* that enables more efficient detection and eliminates or mitigates the problems with existing techniques. It is a further object to provide a method of distinguishing the *campylobacter* species *jejuni, coli, upsaliensis* and *lari*.

Accordingly, in a first aspect the present invention provides a method of testing for the presence of *campylobacter*, e.g. in a clinical, environmental or food sample, comprising the steps of performing polymerise chain reaction (PCR) using primers adapted to amplify a region selected from (a) a sequence of at least 72 base pairs from sequence ID No. 1 and (b) a sequence having sufficient homology with (a) such that formation of PCR product is correlated with presence of *campylobacter*; and determining if any PCR product is formed.

It is preferred that sequence (b) has at least 75% homology with (a), preferably at least 90% homology and more preferably at least 95% homology. It is also preferred that the primers are at least 12 nucleotides in length, preferably between 19-22 nucleotides in length. In particularly preferred embodiments of the invention the primers consist of at least 12 contiguous nucleotides selected from (1) sequence ID NO:2 and sequence ID NO:3, (2) sequence ID NO:4 and sequence ID NO:5 and (3) sequences having sufficient homology with (1) or (2) such that formation of PCR product is correlated with presence of *campylobacter*

PCR has become a well known and established tool for DNA analysis. A single gene sequence can be marketed from a large amount of other DNA and amplified to provide a suitable quantity for analysis. The basis of todays PCR was first published in 1971 be Kleppe, E et al, J. Mol. Biol., 1971, 56, 341. Further significant details and improvements on the PCR

method have been added by Saiki, R K et al, science, 1985, 230, 1350 and Mullis, K B, sci. am. 1990, 262, 36.

As will be appreciated by a person of skill in the art familiar with the PCR, it is important to operate at a temperature suitable to ensure that the primers used are specific for the sequence desired to be identified and amplified. To this end it is convenient to carry out the PCR reaction using the method of the invention at temperatures of at least 40°C preferably at least 45°C and in a particularly preferred embodiment at 48-52°C.

The 1.9 kilobase fragment identified in sequence ID 1 is an underlying feature of this invention and has been found to be highly conserved between *campylobacter* isolates. The method of the invention confers the advantage that PCR product will only be detected when a *campylobacter* strain is found in the sample tested. The method also confers the advantage that it will detect non-culturable viable forms of *campylobacter* as well as viable cells. Thus the method is effective where other methods have not been able to detect any *campylobacter*.

It is preferable to use a primer sequence that will only bind to one specific region of sequence ID NO:1 and which will not engage in formation of primer dimers and thus contaminate the PCR. Examples of preferred primers for use in the method of the invention are shown in the sequence ID numbers 2 and 3 and sequence ID numbers 4 and 5. These primers form further aspects of the invention.

In a second aspect the invention provides a method of distinguishing between *campylobacter* species *jejuni*, *coli*, *upsaliensis* and *lari* in a DNA containing sample by performing PCR utilizing primers capable of amplifying a selected *campylobacter* DNA sequence, said sequence having restriction

endonuclease sites specifically associated with different *campylobacter* species and then testing for digestion of the PCR product by the specific restriction endonucleases.

Thus, campylobacter DNA that is differentially cleared by restriction endonucleases is amplified, subject to digestion by the endonucleases and identified as from a particular species.

Sequence ID NO:1 was isolated from *Campylobacter jejuni* and is known to have a particular characteristic pattern of cleavage by restriction endonucleases. *Campylobacter coli, upsaliensis* and *lari* contain sequences corresponding to sequence ID NO:1 that have altered patterns of cleavage characteristic of each species.

In an embodiment of the second aspect there is provided a method of distinguishing between *campylobacter* species *jejuni, coli, upsaliensis* and *lari*, e.g. in a clinical, environmental or food sample containing *campylobacter*, comprising the steps of:-

performing polymerise chain reaction (PCR) on the sample using primers adapted to amplify a region of DNA sequence ID No.1 that includes nucleotides 124-196, or using primers adapted to amplify a DNA region corresponding thereto; and

testing the PCR product for digestion by restriction endonucleases Alu I, Dra I and Dde I.

The method of the second aspect is advantageous because it enables accurate speciation between the four clinically most significant species. In particular, when amplifying region 124-196 of SEQ DI NO:1, the PCR product from *campylobacter*

jejuni is cleaved by all three restrictions endonucleases, whereas the PCR product from species *coli* is not cleaved by Dra I, the PCR product from species *upsaliensis* is only cleaved by Dde I and PCR product from *lari* is only cleaved by Alu I. It is a straightforward matter for a person skilled in the art to identify whether the PCR product is cleaved by one or more of the above endonucleases and thus the method enables simple speciation of *campylobacter* into *jejuni*, *coli*, *upsaliensis* or *lari*.

The embodiments of the first aspect of the invention described above form embodiments of the second aspect of the invention also, provided that primers are selected so as to be adapted to amplify at least nucleotides 124-196 of sequence ID NO:1, or a *campylobacter* sequence corresponding thereto.

In a preferred embodiment of the second aspect the primers consist of at least 12 contiguous nucleotides from sequence ID NO:s 4 and 5. Where the primers are sequences ID NO:s 4 and 5 the PCR product is 256bp and the respective products of cleavage by Alu I, Dra I and Dde I differentiate between *jejuni*, *coli*, *upsaliensis* and *lari*.

In a further embodiment of the invention, increased sensitivity and specificity for the detection of the presence of Campylobacter DNA, e.g. in food and liquid samples, is provided by the following additional methodologies:

1. A nested PCR has been developed, and is performed by an additional round of amplification using primer sequences international primer sequences ID4 and ID5. Two exemplary primer sequences are identified as Cru 0476 (SEQ ID NO:6) and Cru 0474 (SEQ ID NO:7). Following the second round of amplification, an amplicon of approximately 173pb is obtained in the presence of Campylobacter DNA. This DNA fragment

retains the sequences for the restriction endonucleases Alu 1, Dde 1, and Dra 1, thus still enabling the speciation of the contaminating campylobacter.

2. Additional increased sensitivity and specificity is optionally achieved by southern transfer of the amplified PCR products obtained using *oligonucleotide* primers ID4 and ID5, followed by hybridisation with an internal probe (e.g. SEQ ID NO:8 probe sequence). The probe sequence spans the restriction sites for speciation of the contaminating campylobacter and therefore restriction digest analysis can be used in conjunction with the probe hybridisation to confer additional specificity. The probe can be labelled, for example with digoxigenin, or radiolabelled.

The extraction procedures for food and environmental samples preferably use an internal standard to enable qualitative estimation of extraction efficiency and the effects of non-specific inhibition. The PCR "MIMIC" (Clontech Laboratories, Palo Alto, California) is a form of competitive PCR in which a non-homologous neutral DNA fragment is engineered containing the same primer templates as the target DNA. The amplimer produced from this construct is a fragment either smaller or larger than the target product. Known amounts of construct are added to the PCR reaction, and compete for the same primers, acting as an internal standard. Where a mimic is used, the mimic sequence is capable of being amplified by the same primers that amplify, under PCR conditions, the campylobacter sequence. The mimic, if cleaved by restriction endonucleases, does not form fragments that interfere with detection and/or speciation of campylobacter-the mimic is said to be "neutral".

It is preferred to carry out the PCR steps of the invention also using a mimic. In an example, mimic DNA is added to the

sample and PCR is performed according to the invention. The PCR product is analyzed. If mimic DNA ha been amplified, this indicates that the PCR reaction has occurred properly. The product can then be tested for products that indicate presence of campylobacter. If no mimic DNA is amplified then this indicates PCR has not fully been carried out, or has been inhibited in some way.

It is further preferred to carry out PCR using mimic DNA of known and varying quantities. After amplification, the various results are compared and it is observed which of the results has comparable amounts of amplified mimic and (if present) campylobacter DNA. Thus, an estimate of the quantity of campylobacter DNA in the original sample is obtained.

The methods of the invention are further illustrated by the further embodiments of the invention described in the following Examples:-

Example 1

The PCR assay was developed by the following steps:

1> Identification of a highly conserved, species specific clone from a random library of *Campylobacter jejuni* insert fragments, cloned in the vector pBlueScript KS.

2> Chain termination sequencing of the 1.9 kilobase fragment in both directions.

3> Selection of presumptive primer pairs based on regions of equivalent G+C/A+T content, and low identity (prevention of 'primer-dimer').

4> Optimisation of reaction parameters: Mg^{++} concentration, *Taq* enzyme source, buffer composition, annealing temperature,

cycling parameters.

Example 2

ASSESSMENT OF ASSAY SENSITIVITY AND SPECIFICITY

Using a single amplification (35 cycles, annealing temperature 50°C) we detected approx. 10 CFU/ml of *Campylobacter jejuni*.

We at this stringency, the assay was specific for *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter upsaliensis*. Using a lower annealing temperature (42°C), *Campylobacter* fetus and *Campylobacter* lari were also amplified.

Example 3

The following procedures were used for PCR amplification of *Campylobacter jejuni* from milk and water samples.

1> cell lysis by boiling or freeze/thaw cycles, centrifuge, pcr supernatant directly.

2> Cell lysis by boiling, nucleic acid purification by phenol\chloroform extraction

3> cell lysis by guanidine isothiocyanate, nucleic acid purification using nuclease binding matrix ("isoquick").

4> Cell concentration using magnetic particles coated with anti-campylobacter igg, cell lysis by boiling.

5> concentration and immobilisation of cells on 0.2µm nitrocellulose filters ('solid-phase' pcr).

6> Cell concentration using affinity column purification

7> guanidium isothiocyanate nucleic acid extraction, with purification using silica bead matrix ('boom method')

EXTRACTION OF MILK SAMPLES

FOR PCR ANALYSIS

WARM MILK TO 37°C

↓

CENTRIFUGE @ 3,000xg, 15 MINUTES

↓

CHILL ON ICE. SEPARATE MILK AND CREAM

CREAM	MILK
EMULSIFY IN 10 VOLUMES OF WARM PBS	
↓	↓
CENTRIFUGE @ 9,000xg, 15 MINUTES	
↓	
DISCARD SUPERNATANT	DISCARD SUPERNATANT

RESUSPEND MILK AND CREAM PELLETS IN 5 VOLUMES OF PBS. POOL EXTRACTS.

↓

BOIL FOR 10 MINUTES

↓

CENTRIFUGE @ 14,000xg, 5 MINUTES

↓

EXTRACT DNA WITH SILICA-BASED PURIFICATION MATRIX

↓

ELUTE NUCLEIC ACIDS WITH 2x50µl PURE WATER

↓

PCR NEAT SAMPLE, AND 10-FOLD SERIAL DILUTIONS

EXTRACTION OF WATER SAMPLES

PRE-FILTRATION THROUGH 30µm WHATMAN FILTER
↓
CENTRIFUGE @ 9,000xg, 15 MINUTES
↓
WASH PELLET x2, 1ML PBS
↓
RESUSPEND IN 1ML STERILE WATER
↓
BOIL, 10 MINUTES
↓
EXTRACT DNA WITH SILICA-BASED PURIFICATION MATRIX

Example 4

We observed the following differentiation of Campylobacter species using PCR primes SEQ ID NO:4 and SEQ ID NO:5 and restriction endonucleases Alu I, Dra I and Dde I.

	PCR product digested with:		
Species	*Alu* I	*Dra* I	*Dde* I
C. jejuni	+	+	+
C. coli	+	-	+
C. upsaliensis	-	-	+
C. lari	+	-	-

We further observed the following fragment sizes for different species.

Species	Restriction enzyme digests of PCR amplimers					
	Alu I		Dde I		Dra I	
Thermophilic/ enteropathogenic		Fragment sizes (bp)		Fragment sizes (bp)		Fragment sizes (bp)
C. jejuni	2	108, 148	2	83, 173	2	123, 133
C. jejuni (hippurate + ve)	2	108, 148	2	83, 173	2	123, 133
C. coli	2	108, 148	2	83, 173	1	256
C. lari	2	108, 148	1	256	1	256
C. upsaliensis	1	256	3	30,83,143	1	256

The results are also illustrated in Fig. 3 where bonds were not visible by eye they were detected by use of radiolabels.

Example 5

To test the specificity of campylobacter detection we used PCR primers on laboratory samples containing a wide range of organisms. The primers were SEQ ID NO:s 4 and 5, PCR product size in brackets:

	Annealing temperature of primers		
Species	37°C	42°C	50°C
C. jejuni	+ (256)	+	+
C. coli	+	+	+
C. upsaliensis	+	+	+
C. fetus	+	±	-
C. lari	+	±	-
C. mucosalis	±	-	-
C. sputorum	±	-	-
Achromobacter sp.	-	-	-
Acinetobacter calcoac.	± (multiple)	-	-
Acinetobacter sp.	± (multiple)	-	-
Aeromonas hydrophila	-	-	-
Citrobacter freundii	-	-	-
Enterobact. aerogenes	-	-	-
Enterobact. agglomerans	± (500)	-	-
Enterobacter cloacae	-	-	-
Escherichia coli	-	-	-
Flavobacterium	-	-	-

Species	37°C	42°C	50°C
Klebsiella aerogenes	-	-	-
Klebsiella oxytoca	± (500)	-	-
Proteus mirabilis	-	-	-
Proteus morganii	-	-	-
Providencia stuartii	-	-	-
Pseudomonas aeroginosa	-	-	-
Pseudomonas maltophilia	-	-	-
Pseudomonas pickettii	-	-	-
Salmonella enteritidis	-	-	-
Salmonella typhimurium	-	-	-
Serratia marcescens	-	-	-
Serratia liquefaciens	-	-	-
Shigella dysenteriae	-	-	-
Shigella sonnei	-	-	-
Vibrio cholera	-	-	-
Vibrio furnassii	± *(1000)*	-	-
Vibrio parahaemolyticus	± *(180)*	±	-
Yersinia enterocolitica	-	-	-
Oxford staphlococcus	± (300)	±	-

Example 6

Using standard culture techniques (published by Bolton F J, et al, J. Appl. Bacteriol., 1983, vol. 54, pages 115-125) we compared the detection of *campylobacter jejuni* by culture with detection by the method of the invention (using primers SEQ ID NO:s 4 and 5) against time.

The success of culture detection declined over the time of the comparison, no culturable organisms being found remaining in the sample after 26 days - thus at this point detection by culture indicated no campylobacter present.

By contrast, using the PCR method of the invention we were still able to detect campylobacter DNA in a sample 42 days old. The results are illustrated in Figure 1.

Example 7

To confirm the accuracy of the PCR method of the invention we tested many samples that contained known species of campylobacter. The results, illustrated in figs. 4-11, confirm the method is completely accurate for all samples tested, and correctly identified each one by species.

FEATURES OF THE PCR ASSAY FOR *Campylobacter jejuni*

It allows rapid and sensitive detection of *Campylobacter jejuni* from environmental samples,

provides a semi-quantitative indication of the bacterial load, and determines whether samples are contaminated with *Campylobacter jejuni, coli, upsaliensis* or *lari*.

The method is of use for examining epidemiology of *campylobacter* infection such as a) seasonal peak, b) inverse correlation of surface water viable counts with human disease, c) role of water supply in (re)infection of broiler flocks with *Campylobacter jejuni*, d) contamination of foodstuffs at the point of sale, and e) determine origin of sporadic human infections.

Thus, a novel method incorporating polymerise chain reaction assay has been developed for the detection of *campylobacter* in clinical, environmental and food samples, such as milk and water samples. The assay is rapid, highly sensitive, and specific for *Campylobacter* sp. Simple restriction analysis of the PCR product allows speciation between *Campylobacter jejuni, coli upsaliensis* and *lari*.

Description of Drawings

Fig. 1 shows a comparison of culturability of *campylobacter jejuni* against time with detection of *campylobacter jejuni* using PCR of the invention;

Fig. 2 shows the sequence of open reading frame "C" from insert fragment pBSKSCJ19B with primer/nested primer locations, and restriction sites;

Fig. 3 shows restriction enzyme analysis of PCR products amplified from *C. jejuni, coli* and *upsaliensis*.

Fig. 4-11 shows the results of carrying out the PCR method of the invention on samples containing a wide ranges of known isolates. "P"=Penner Serotype Reference strains. "L"=Lior Serotype Reference Strains. Others are laboratory isolates. Standard size markers are on the gel ends.

Fig. 4	*C. Jejuni.*	Alu I digest.
Fig. 5	*C. Jejuni.*	Dde I digest.
Fig. 6	*C. Jejuni.*	Dra I digest.
Fig. 7	*C. upsaliensis.*	Alu I and Dde I digest.
Fig. 8	*C. upsaliensis.*	Dra I digest.
Fig. 9	*C. Coli.*	Alu I digest
Fig. 10	*C. Coli.*	Dde I digest
Fig. 11.	*C. Coli.*	Dra I digest

Sequence ID No. 1

```
   1 accaacagcc attaaaaatc ttgactcagc catactcact ttaagaacac
     tggttgtcgg taatttttag aactgagtcg gtatgagtga aattcttgtg

  51 gcggacctat ataataccgt tgcccaaatc cctgaaagca taaaaccaaa
     cgcctggata tattatggca acgggtttag ggactttcgt attttggttt

 101 aatcacacct gaagtatgaa gtggtctaag tcttgaaaaa gtggcatatt
     ttagtgtgga cttcatactt caccagattc agaacttttt caccgtataa

 151 gtcctggtaa ataatttaaa ttaggatatg ccatttgaaa agctataaga
     caggaccatt tattaaattt aatcctatac ggtaaacttt tcgatattct

 201 gttcctatag ccataccaac aatgccaaac aatatggtcg caaacataaa
     caaggatatc ggtatggttg ttacggtttg ttataccagc gtttgtattt

 251 atatcttgca accgtatagt cgtaatttaa tacattacct ggatgcatcg
     tatagaacgt tggcatatca gcattaaatt atgtaatgga cctacgtagc

 301 actttctcct taaaattttt gataacaaga gaagattata gaatattaat
     tgaaagagga attttaaaaa ctattgttct cttctaatat cttataatta

 351 tatacatttt ttcttaaaaa tgat/aatttt gttaatcatt tgttatgttt
     atatgtaaaa aagaattttt acta ttaaaa caattagtaa acaatacaaa

 401 tatattttaa ggctaaatca gtcttattta ttgatattta tcttataacc
     atataaaatt ccgatttagt cagaataaat aactataaat agaatattgg

 451 taaacttgtc acatttttta taaaatcttc acccacttta tctcttactc
     atttgaacag tgtaaaaaat attttagaag tgggtgaaat agagaatgag

 501 tttttataaa agttcta{aca gcagtatcgc tcacatgt}c acctatccaa
     aaaaatattt tcaagat tgt cgtcatagcg agtgtaca g tggataggtt

 551 acatttttct taatatcttc atgcaaaacc aaagctccag gttgctttaa
     tgtaaaaaga attatagaag tacgttttgg tttcgaggtc caacgaaatt

 601 aagcaaagaa ataaaagcca attctttttt agttaaaaca atttctccac
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     gtgacattta atttcaagca aataaaaaca atttaactat aagaagtctt

 701 atttttacaa gcatatttgc ttcaattttt tcacctatca gataatctaa
     taaaaatgtt cgtataaacg aagttaaaaa agtggatagt ctattagatt

 751 aactttaaac aactcttcta tatcaacagg tttaatcaaa tatttatcta
     ttgaaatttg ttgagaagat atagttgtcc aaattagttt ataaatagat
```

Sequence ID No. 1 (cont)

801 taccaatatc aatagaacgc aaaagtctct ctt tctctga atacgcacta
atggttatag ttatcttgcg ttttcagaga gaa{agagact tatgcgtgat

851 aga acaacaa ttgggacatc atctgaaatt tctttaatct ctcttgccat
tct tgttgaa aaccctgtag tagactttaa agaaattaga gagaacggta

901 atccagtcca tccataatag gcatagcaat atctgtgata actaaatctg
taggtcaggt aggtattatc cgtatcgtta tagacactat tgatttagac

951 gcttaaattt tttaaatttt ttaagcccct catctccatt ttgagctccg
cgaatttaaa aaatttaaaa aattcggggt gtagaggtaa aactcgaggc

1001 attactttac taaagcgttc gcttaatata ttaatcattg attctctagc
taatgaaatg atttcgcaag cgaattatat aattagtaac taagagatcg

1051 cttaacctca tcttcaacta ctaatattat taattcttta cattcttgtg
gaattggagt agaagttgat gattataata attaagaaat gtaagaacac

1101 acat/ttctac tctaccctct cttttagttt taaaaatatc tcaaaacaag
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1151 ccccgtcttt tccattttta acttttattt ttccttggaa actttcgata
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1301 caccagcatt atcttttatt gtaattttca gataattttt tccaaatttt
gtggtcgtaa tagaaaataa cattaaaagt ctattaaaaa aggtttaaaa

1351 gaaaaattta ttgttatgat tttccttttt ttgtttttaa atgcttctat
ctttttaaat aacaatacta aaaggaaaaa aacaaaaatt tacgaagata

1401 tgaatttaaa atcaaattaa gaaaaactct tattaaacca ttctcatatg
acttaaattt tagtttaatt ctttttgaga ataatttggt aagagtatac

1451 ccaaaacttc ataatcactt ttcgaaacaa tattaatatt tacatgattt
ggttttgaag tattagtgaa aagctttgtt ataattataa atgtactaaa

1501 ttttctatag tttcaaaagc aatttccaag gctttattta aagtctcttt
aaaagatatc aaagttttcg ttaaaggttc cgaaataaat ttcagagaaa

1551 tataaataca cactgctcta ctcctttgtt aaacaaagtt ctaaacacat

1601 caattgtttc tgacatattt ttaatcatat cttttgattg tgagtaaatt
gttaacaaag actgtataaa aattagtata gaaaactaac actcatttaa

1651 tcagcaaatc ctttttcatc tttaagattt tgcttcattt gaaacatggc
agacgtttag gaaaaagtag aaattctaaa acgaagtaaa ctttgtaccg

Sequence ID No. 1 (cont)

1701 aataccgagc tcatttaacg gttgtctcca ttgatgtgct atatcactaa
ttatggctcg agtaaattgc caacagaggt aactacacga tatagtgatt

1751 tcatttgttc taatgaagat ttcaaaatct cttcatatgc tattttaata
agtaaacaag attacttcta aagttttaga gaagtatacg ataaaattat

1801 tctttttcat ttttttccaa ggcaatttgc atttttttct caaatttttt
agaaaaagta aaaaaaggtt ccgttaaacg taaaaaaaga gtttaaaaaa

1851 acctaactgt ataaattctt gttggtgatt tttaactgta ttttcaagat
tggattgaca tatttaagaa caaccactaa aaattgacat aaaagaacta

1901 taatacttaa ttctcttaat ttagcgtgat ttagagcaag ctcttcat
attatgaatt aagagaatta aatcgcacta aatctcgttc gagaagta

Sequence ID No. 2

5' TCTTAGTGCG TATTCAGAGA 3'

Sequence ID No. 3

5' ACAGCAGTAT CGCTCACATG T 3'

Sequence ID No. 4

5' AGAACACGCG GACCTATATA 3'

(also referred to as B04263)

Sequence ID No. 5

5' CGATGCATCC AGGTAATGTA T 3'

(also reffered to as B04264)

Primer Sequences

Internal

Cru 0476 (SEQ ID NO: 6)
5' a t c a c a c c t g a a g t a t g a 3' 18 mer

Cru 0474 (SEQ ID NO: 7)
5' t a c g a c t a t a c g g t t g c a 3' 18 mer

Amplimer size: 173 base pairs

SEQ ID NO: 8

Probe sequence (173 bp amplimer from nested primers)

```
tacgactatacggttgcaagatattttatgtttgcg
-----+---------+---------+---------+
atgctgatatgccaacgttctataaaatacaaacgc

Y  D  Y  T  V  A  R  Y  F  M  F  A

     accatattgtttggcattgttggtatggct
     ---------+---------+---------+1740
     tggtataacaaaccgtaacaaccataccga
     T  I  L  F  G  I  V  G  M  A  -


                  Alu I
     ataggaactcttatagcttttcaaatggca
1741 ---------+---------+---------+
     tatccttgagaatatcgaaaagtttaccgt
     I  G  T  L  I  A  F  Q  M  A  -


            Dra I
     tatcctaatttaaattatttaccaggacaa
     ---------+---------+---------+1800
     ataggattaaatttaataaatggtcctgtt
     Y  P  N  L  N  Y  L  P  G  Q  -


                     Dde I
     tatgccacttttttcaagacttagaccacttcatacttcaggtgtgat
1801 ---------+---------+---------+---------+-------
     atacggtgaaaaagttctgaatctggtgaagtatgaagtccacacta

     Y  A  T  F  S  R  L  R  P  L  H  T  S  G  V  I
```

PCR MIMIC primers and sequence

Primer 1 Cru 0477 (SEQ ID NO: 9)

5' agaacacgcggacctatatacgcaagtgaaatctcctccg 3' 40 mer

Primer 2 Cru 0660 (SEQ ID NO: 10)

5' cgatgcatccaggtaatgtattctgtcaatgcagtttgtag 3' 41mer

MIMIC SEQUENCE (SEQ ID NO: 11)

5' agaacacgcg gacctatata cgcaagtgaa atctcctccg
tcttggagaa gggagagcgt ttgccccagc taccattgat
gtgtacatga tcatggtcaa atgctggatg attgatgcag
acagccgtcc caagtttcgt gagctgattg cagagttctc
caaaatggct cgtgaccctc cccgctatct tgttatacag
ggagatgaaa ggatgcactt gcctagccct acagattcca
agttttatcg caccctgatg gaggaggagg acatggaaga
cattgtggat gcagatgagt atcttgtccc acaccagggc
tttttcaaca tgccctctac atctcggact cctcttctga
gttcattgag cgctactagc aacaattctg ctacaaactg
cattgacaga 3'

CLAIMS:

1. A method of testing for the presence of *campylobacter* in a clinical, environmental or food sample comprising the steps of:-

performing polymerise chain reaction (PCR) using primers adapted to amplify a region selected from (a) a sequence of at least 72 base pairs from sequence ID No. 1 and (b) a sequence having sufficient homology with (a) such that formation of PCR product is correlated with presence of *campylobacter*; and determining if any PCR product is formed.

2. A method according to claim 1 in which sequence (b) has at least 75% homology with (a), preferably at least 90% homology and more preferably at least 95% homology.

3. A method according to Claim 1 or 2 in which the primers comprise at least 12 nucleotides.

4. A method according to Claim 3 in which the primers are 19-22 nucleotides in length.

5. A method according to Claim 3 or Claim 4 in which the primers consist of at least 12 nucleotides selected from:

(1) sequence ID No.2 and sequence ID No.3,
(2) sequence ID No.4 and sequence ID No.5, and
(3) sequences having sufficient homology with (1) or (2) such that formation of PCR product is correlated with presence of *campylobacter*.

6. A method according to any previous claim in which the temperature of the PCR is sufficiently high to prevent the primers annealing with non-*campylobacter* DNA.

7. A method according to Claim 6 in which the PCR temperature is at least 40°C.

8. A method according to Claim 7 in which the PCR temperature is at least 45°C.

9. A method according to Claim 8 in which the PCR temperature is 48-52°C.

10. A method of distinguishing between *campylobacter* species *jejuni, coli, upsaliensis* and *lari* in a DNA containing sample by performing PCR utilizing primers capable of amplifying a selected *campylobacter* DNA sequence, said sequence having restriction endonuclease sites specifically associated with the different *campylobacter* species, obtaining PCR product and then testing for digestion of the PCR product by the specific restriction endonucleases.

11. A method according to claim 10 wherein the selected *campylobacter* sequence is differentially cleaved by the restriction endonucleases Alu I, Dra I and Dde I

12. A method of distinguishing between *campylobacter* species *jejuni, coli* and *upsaliensis* comprising the steps of:-

performing polymerise chain reaction (PCR) using primers adapted to amplify a region of DNA sequence ID No.1 that includes nucleotides 124-196, or using primers adapted to amplify a region of *campylobacter* DNA corresponding thereto; and

testing the PCR product for digestion by restriction endonucleases Alu I, Dra I and Dde I.

13. A method according to Claim 10, 11 or 12 in which *campylobacter jejuni* is characterised by cleavage by all three endonucleases, *coli* is characterised by loss of cleavage by Dra I endonucleases, *upsaliensis* is characterised by loss of cleavage by Alu I and Dra I endonucleases and *lari* is characterised by cleavage by Alu I only.

14. A method according to any of Claims 10 - 13 characterised by the features of any of claims 3-9.

15. Use of restriction endonucleases Alu I, Dra I and Dde I in differentiating between *campylobacter* species *jejuni, coli upsaliensis* and *lari*.

16. A PCR primer having the sequence of sequence ID No. 2.

17. A PCR primer having the sequence of sequence ID No. 3.

18. A PCR primer having the sequence of sequence ID No. 4.

19. A PCR primer having the sequence of sequence ID No. 5.

20. A PCR primer having the sequence of sequence ID No. 6, 7, 9 or 10.

21. A kit for detecting *campylobacter* comprising one or more reagents for carrying out the method of any of claims 1-9.

22. A kit for determining *campylobacter* species comprising one or more reagents for carrying out the method of any of Claims 10-15.

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CULTURABILITY AND PCR DETECTION OF C. JEJUNI IN A POND WATER MICROCOSM

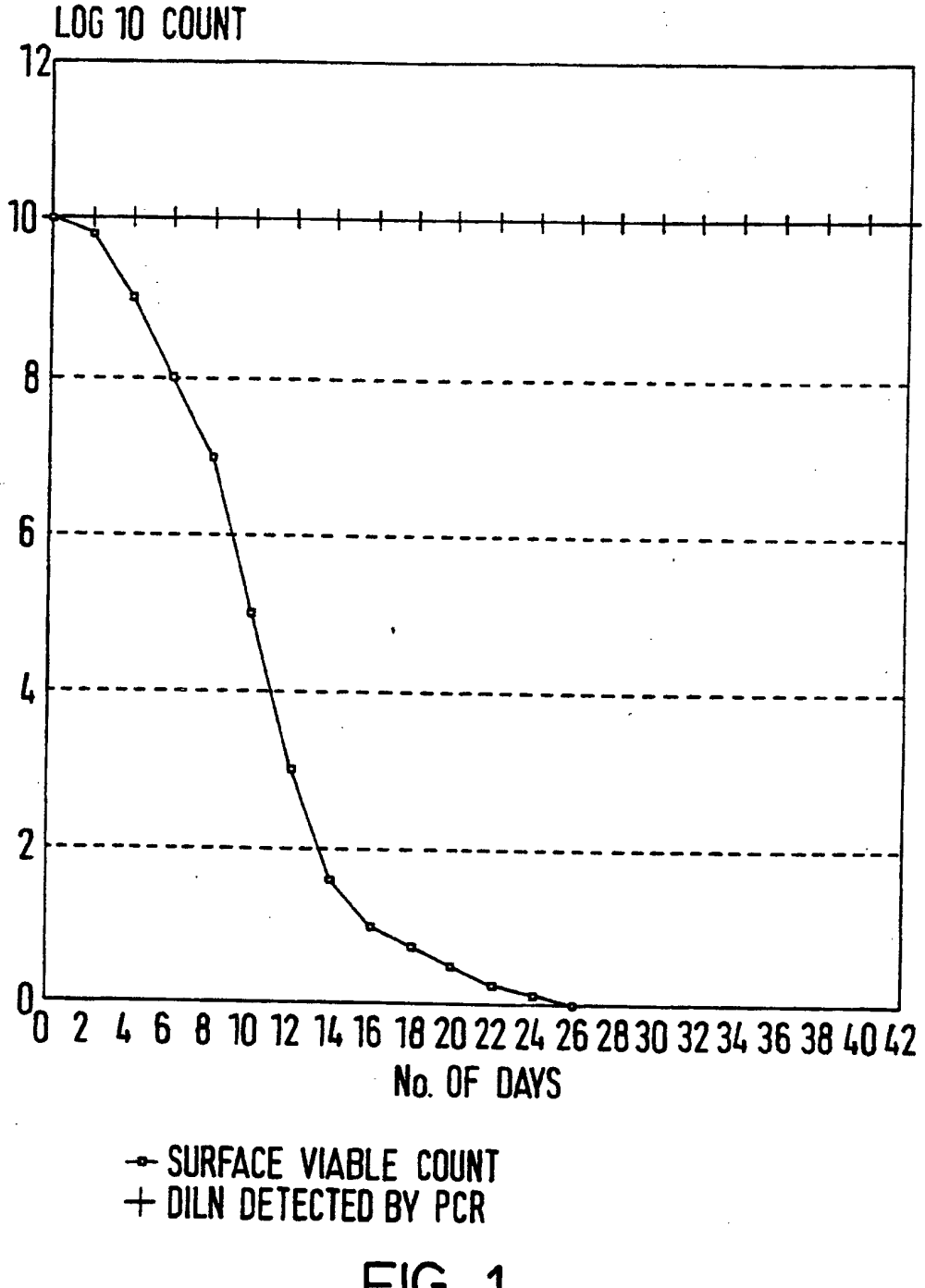


FIG. 1

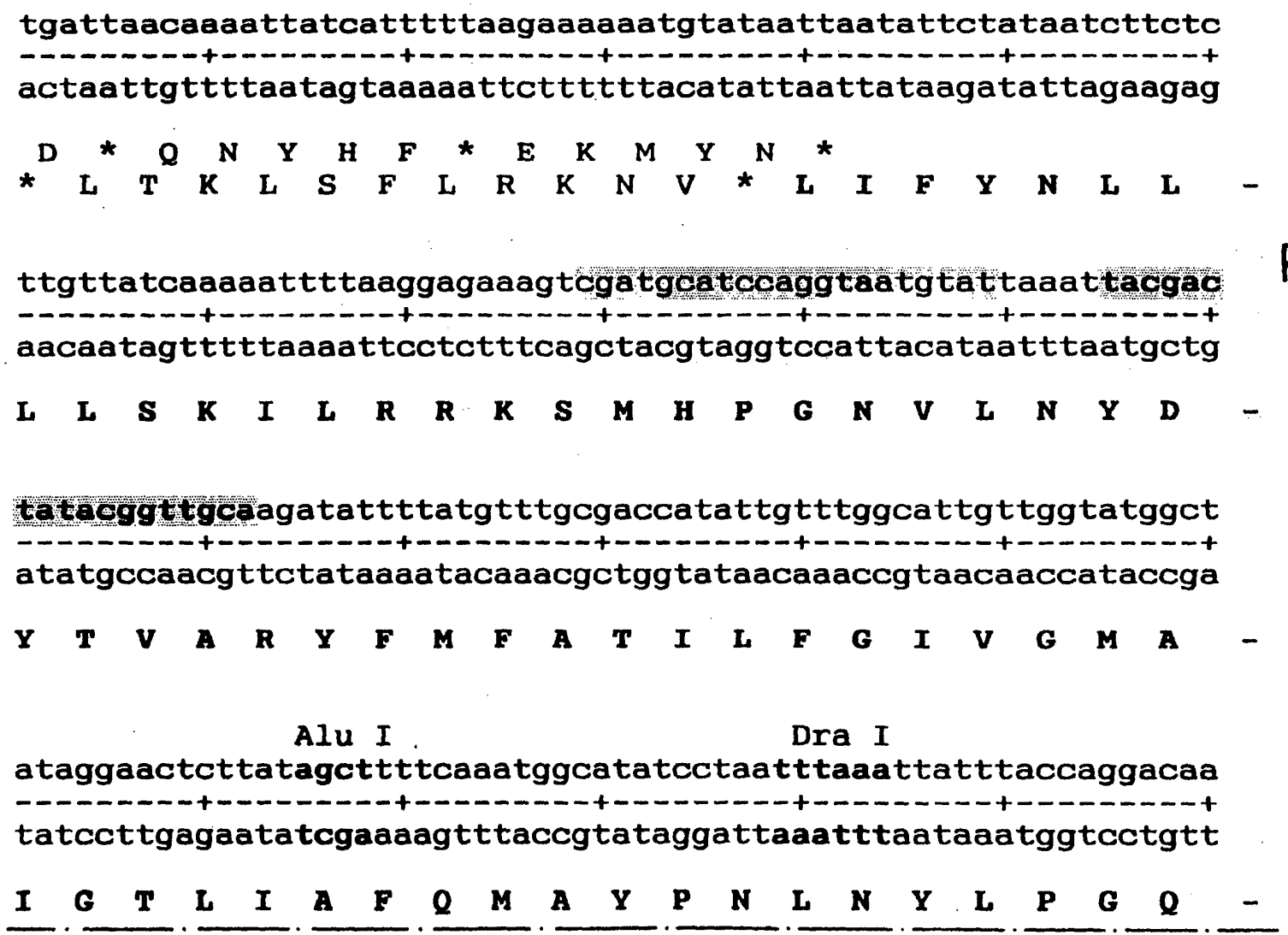
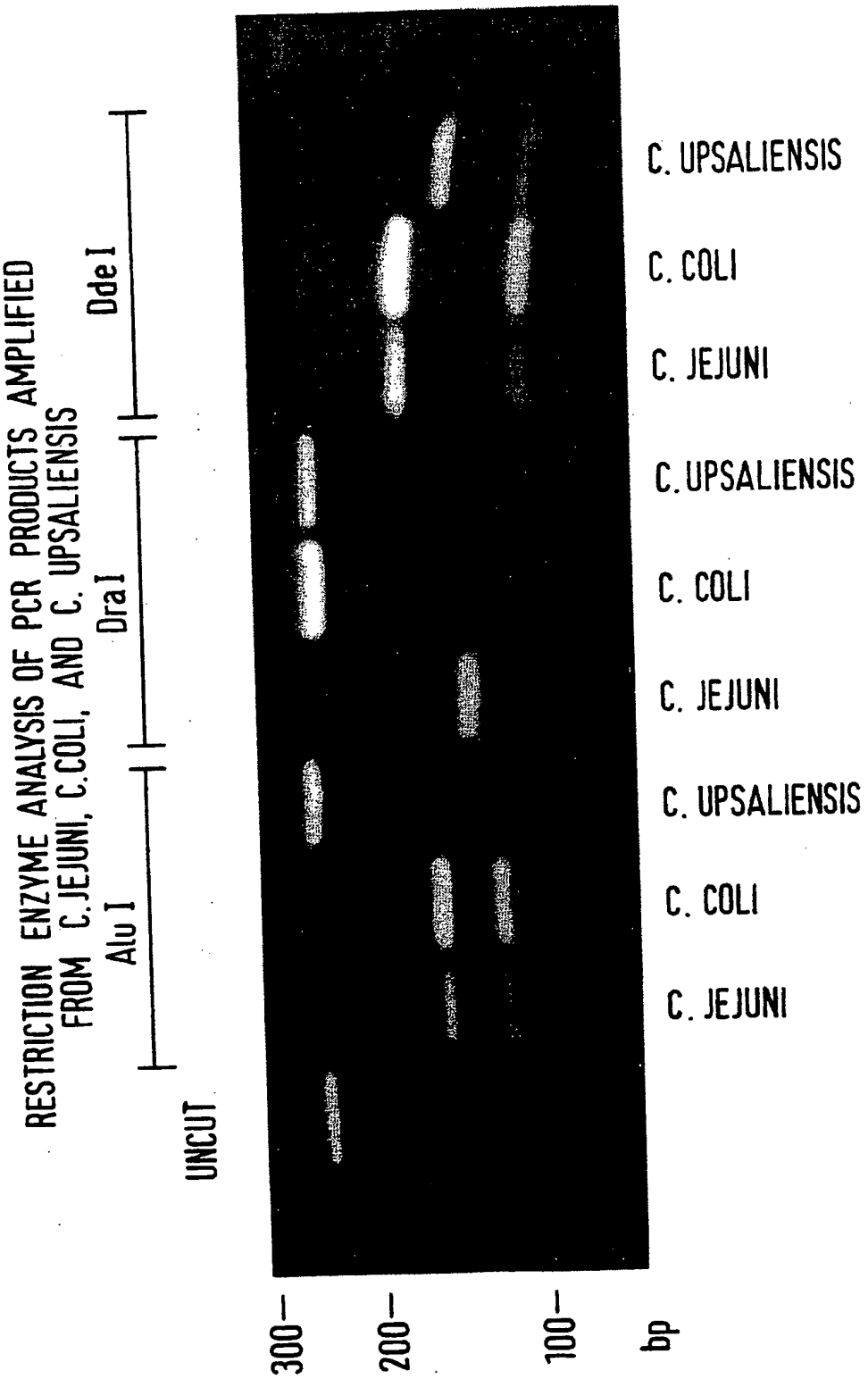


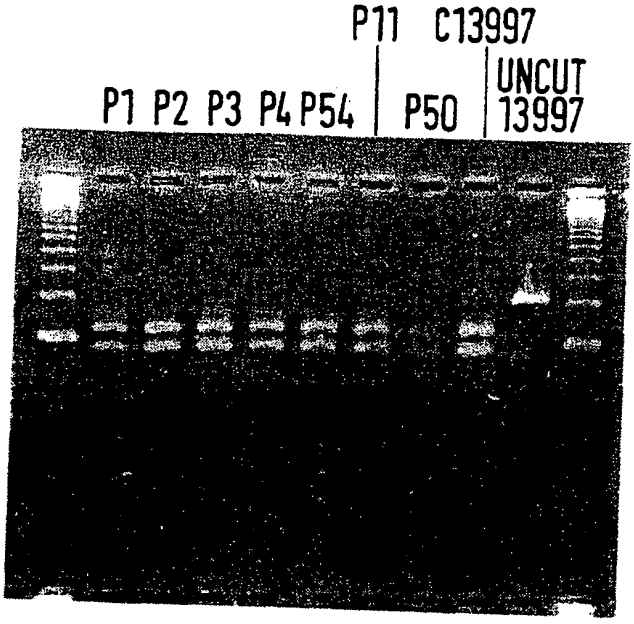
FIG. 2(I)

```
                    Dde I
tatgccactttttcaagacttagaccacttcatacttcaggtgtgattttttggttttatg
----------+----------+----------+----------+----------+----------+
ataccggtgaaaaagttctgaatctggtgaagtatgaagtccacactaaaaaccaaaatac

Y  A  T  F  S  R  L  R  P  L  H  T  S  G  V  I  F  G  F  M  -

ctttcagggatttgggcaacggtattatataggtccgcgtgttcttaaagtgagtatggc
----------+----------+----------+----------+----------+----------+
gaaagtccctaaacccgttgccataatatatccaggcgcacaagaatttcactcataccg

L  S  G  I  W  A  T  V  L  Y  R  S  A  C  S  *  S  E  Y  G  -

tgagtcaagatttttaatggctgttggt
----------+----------+-------- 1948
actcagttctaaaaattaccgacaacca

*  V  K  I  F  N  G  C  W  -
```

FIG. 2(II)

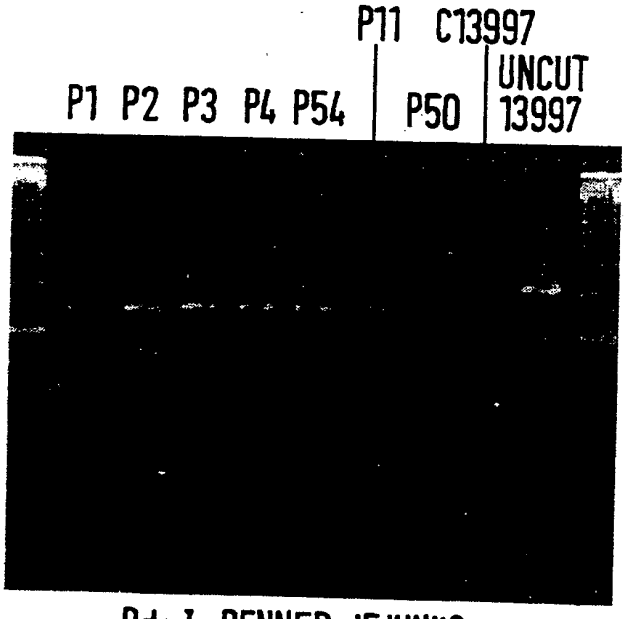
FIG. 3


RESTRICTION ENZYME ANALYSIS OF PCR PRODUCTS AMPLIFIED FROM C.JEJUNI, C.COLI, AND C. UPSALIENSIS
Alu I
Dra I
Dde I
UNCUT
C. UPSALIENSIS
C. COLI
C. JEJUNI
C. UPSALIENSIS
C. COLI
C. JEJUNI
C. UPSALIENSIS
C. COLI
C. JEJUNI
300
200
100
bp



PENNER JEJUNI'S Alu I DIGEST

FIG. 4
SIZE MARKERS ON ENDS



Dde I PENNER JEJUNI'S

FIG. 5

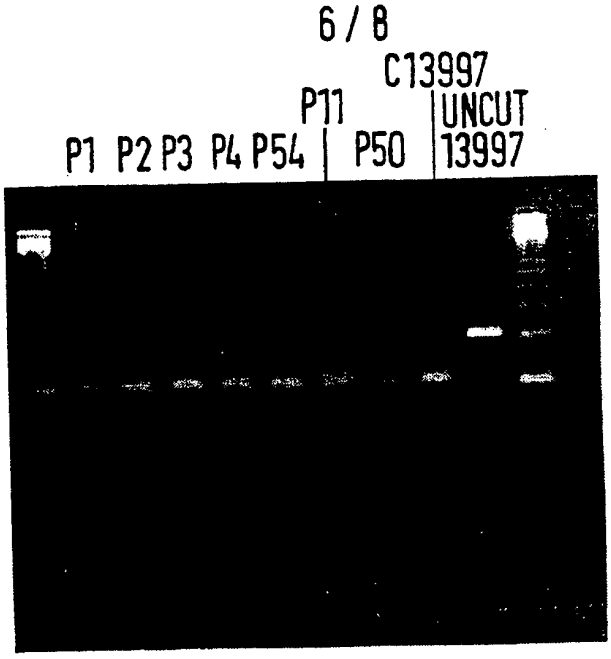

C13997
P11
UNCUT
P1 P2 P3 P4 P54
P50
13997
Dra I PENNER JEJUNI'S

FIG. 6

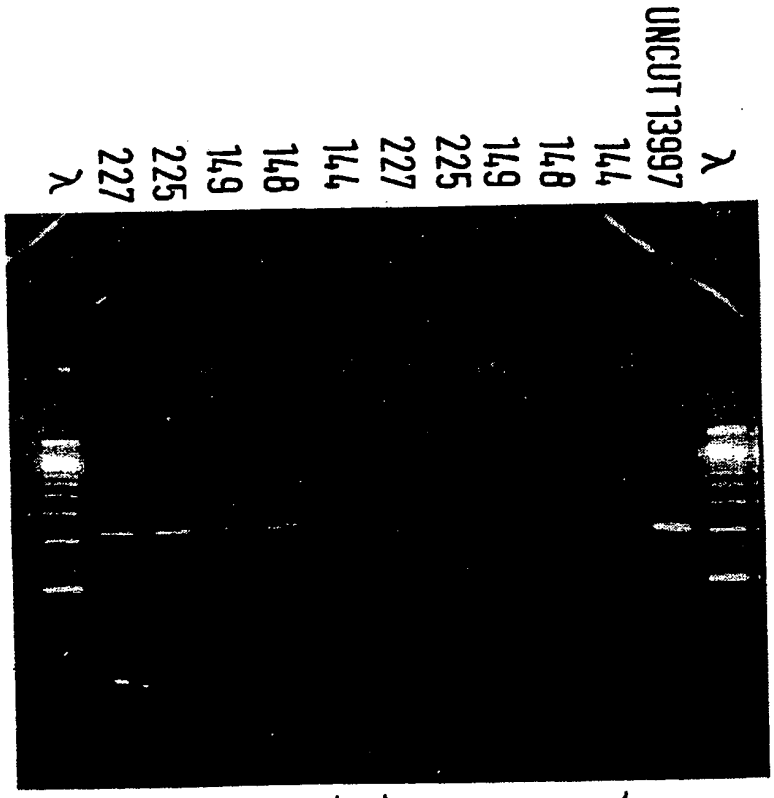

λ 227 225 149 148 144
227 225 149 148 144 UNCUT 13997 λ
Alu I
Dde I
UPSALIENSIS PCR's Alu I Dde I

FIG. 7

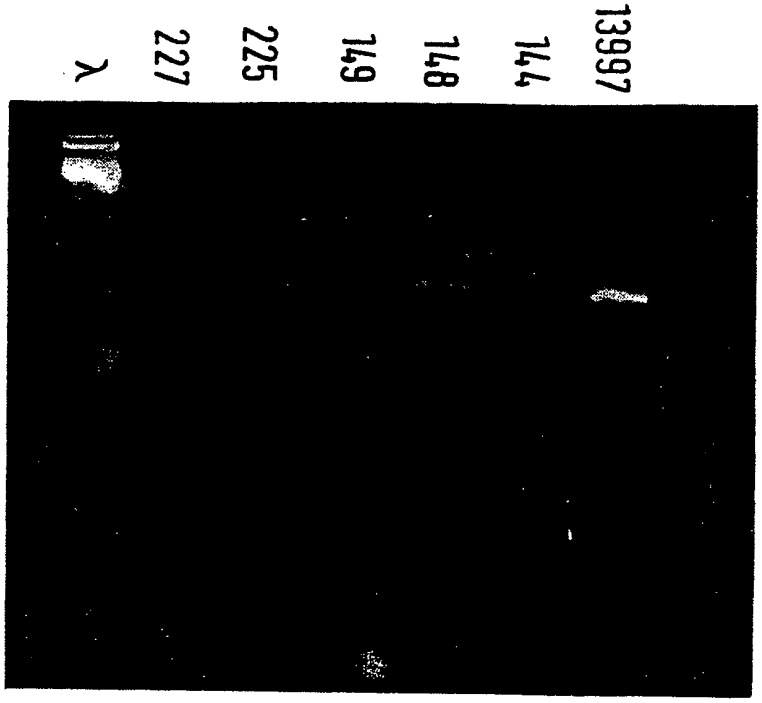


FIG. 8

UPSALIENSIS PCR's Dra I CUT

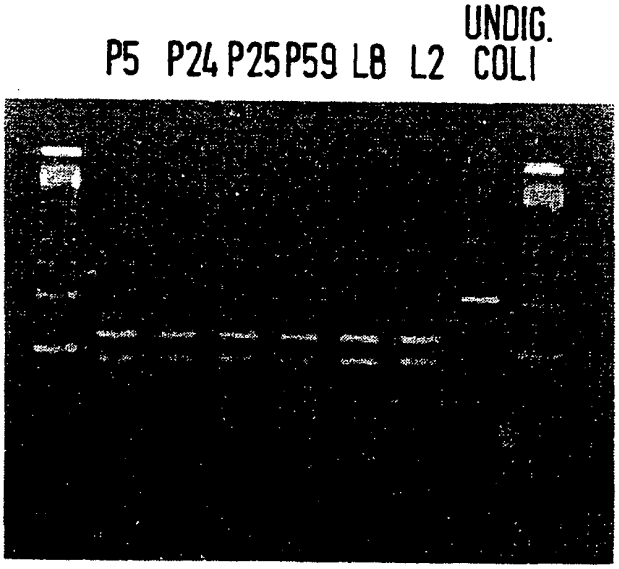
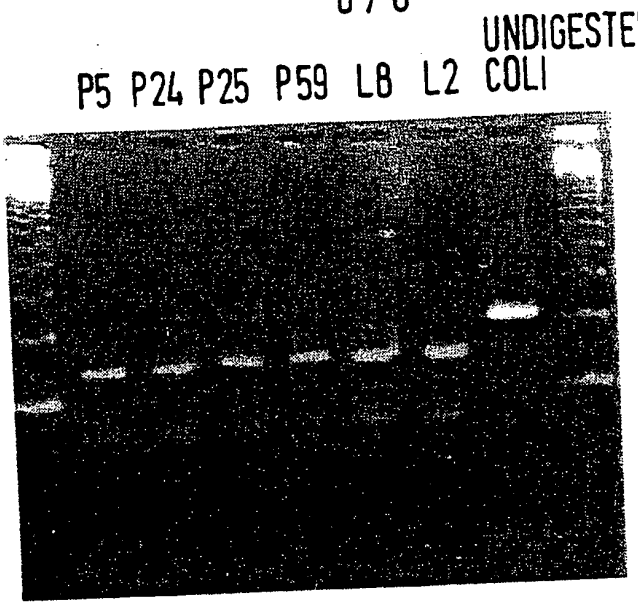


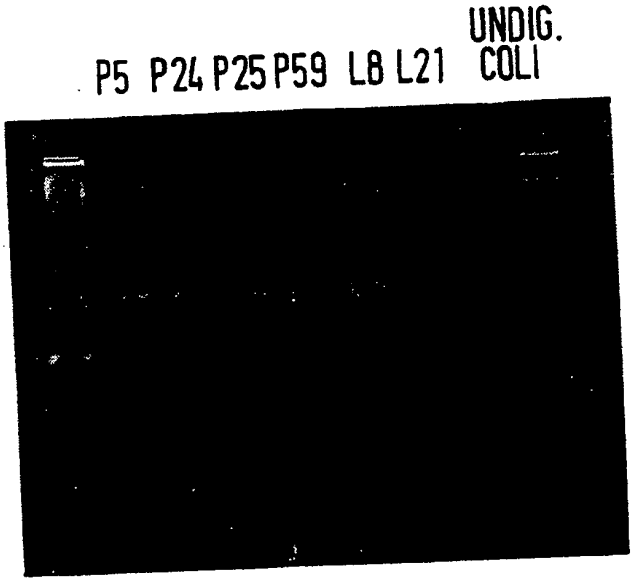
FIG. 9

Alu I - PENNER / LIOR COLI'S



C. Coli Dde I

FIG. 10



Dra I PENNER / LIOR COLI'S

FIG. 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 94/01967

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J CLIN MICROBIOL 31 (6). 1993. 1531-1536. NACHAMKIN I et al 'FLAGELLIN GENE TYPING OF CAMPYLOBACTER -JEJUNI BY STRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS.' see the whole document ---	10-15
X	EP,A,0 350 392 (IRE-MEDGENIX S.A.) 10 January 1990 see claims 12,13; example 5 ---	15
A	LETTERS IN APPLIED MICROBIOLOGY 17 (5). 1993. 235-237. Birkenhead D et al 'PCR for the detection and typing of campylobacters.' --- -/--	10-15

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 January 1995	10.02.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Molina Galan, E

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 94/01967

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J BACTERIOL 175 (10). MAY, 1993. 3051-3057. ALM R A et al 'DISTRIBUTION AND POLYMORPHISM OF THE FLAGELLIN GENES FROM ISOLATES OF MPYLOBACTER -COLI AND CAMPYLOBACTER -JEJUNI.' see the whole document ---	10-15
A	APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol.58, no.12, December 1992 pages 3804 - 3808 GIESENDORF ET AL. 'Rapid and sensitive detection of C. spp. in chicken products by using PCR' cited in the application -----	

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/GB 94/01967

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0350392	10-01-90	FR-A-	2633941	12-01-90
		JP-A-	2154700	14-06-90